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PROTOCOL

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A New Improved Protocol to Obtain Pure Genomic DNA from *Plectranthus forskohlii*(Willd.) Briq.

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Abstract

The mint family Lamiaceae comprising medicinally important plants and economically most wanted inviting its wide cultivation. The improvement in terms of quantity and quality of these plants characters could be possible through molecular techniques which first necessitates the isolation of pure form of DNA. The isolation of plant DNA from *Plectranthus forskoholii* possess great problem because of its high phenolic content together with certain polysaccharide content. In *Plectranthus forskohlii* the extraction of DNA through by means of available methods or commercial kits does not yield pure form of genomic DNA from any parts of the plant species . The present study aims to standardize a DNA isolation procedure to obtain pure genomic DNA from *Plectranthus forskohlii,*. This study includes plant DNA isolation method along with some commercial kit was used to observe their efficiency in isolating pure DNA from the above mentioned plants . The DNA extracted by these three methods from fresh young leaf tissue of above mentioned species were analysed according to their cost and time, yield, purity, integrity and PCR (Polymerase Chain Reaction) based downstream analysis. After the evaluation, one most suitable modified method was selected and chosen for isolating DNA from young leaves of *Plectranthus* species. The cost and time required in this method was relatively low. In addition, the quantity and quality of the DNA extracted by this method were high enough to perform hundreds of PCR based reactions.

Key words: *Plectranthus*; Genomic DNA; Isolation; Polymerase Chain Reaction

Introduction

The field of genomics and its application to plant breeding are developing very quickly. The combination of conventional breeding techniques with genomic tools and approaches is leading to a new genomics-based plant breeding. One of the main pillars of genomic breeding is the development of standard protocol for the isolation of DNA from the plant tissue which forms the basic material subjected to different molecular breeding techniques. Various molecular techniques like PCR and restriction enzyme digestion reaction require isolation of genomic DNA of suitable purity. Successful extraction of DNA that can be amplified by PCR and digested by restriction enzymes can lead to the establishment various molecular approaches. The basic idea behind the DNA extraction is not very complicated, growing number of DNA isolation protocols for specific plant species suggests that the extraction procedures are not always simple and the published protocols are not necessarily reproducible for all species. For those working with medicinal or unexploited crop species, where specific DNA extraction protocol has not been established, the extraction to reliable quantity and purity is a challenging job. The only way is to try various published protocols and do necessary modifications, so that a suitable protocol can be designed. This process is both time consuming and expensive. The DNA extraction process involves separation of DNA from naturally occurring plant cell constituents such as polysaccharides and phenolic compounds which always interfere with isolation and purification of DNA. The present attempt was aimed to isolated the pure form of DNA from *Plectranthus forskohlii* which are most important in medicinal botany and agriculture.

The members of Lamiaceae family contains high phenolic content together with certain polysaccharide content together with certain polysaccharide content makes the isolation of high quality pure genomic DNA problematic. Nevertheless, several protocol for DNA isolation from plant species containing high phenols and polysaccharides have been developed. However, published procedures tested were inadequate for the extraction of high quality DNA from one member of Lamiaceae, *Plectranthus forskohlii* which is commercially and medicinally potential plant.

The whole plant of *P.forskohlii* (root, leaves, Stem) has commercial importance. The plant contains 0.05-0.1% forskolin g^{-1} fresh weight which is a diterpenoid. Roots are the major source of forskolin (Coleonol), although diterpenoids are found in almost all parts (Chandel and Sharma 1997). Leaves also contain diterpenoid methylene quinine, coleon, barabtusin and cyclobutatusin. In the present study, an attempt was made to isolate contaminant free DNA from fresh leaves of *P.forskohlii* with good yield and quality, which can be used for various molecular studies

Problems arising of DNA isolation from *Plectranthus*

The problems encountered in the isolation and purification of DNA specially from the species of *Plectranthus* include degradation of DNA due to endonucleases, co isolation of highly viscous polysaccharides, inhibitor compounds like polyphenols, other secondary metabolites which directly or indirectly interfere with the enzymatic reactions. Moreover, the contaminating RNA that precipitates along with DNA causes many problems including

suppression of PCR amplification (Pikkart&Villeponteau 1993), interfere with DNA amplification involving random primers (Mejjad et al 1994) and improper priming of DNA templates during thermal cycler sequencing.

Different plant taxa often may not permit optimal DNA yields from one isolation protocol. For example, some closely related species of the same genus require different isolation protocols. Thus, an efficient protocol for isolation of pure form of DNA for further molecular techniques. Various protocols for DNA extraction have been successfully applied to many plant species (Doyle & Doyle 1987 &Kathiresan et al 1993), which were further modified to provide pure form of DNA suitable for several kinds of analyses (Sahu et al 2012). We have tested previously established DNA isolation protocols but these methods resulted in DNA with lot of impurities and not very suitable for RAPD analysis.

Therefore, we report here a total genomic DNA of *P.forskohlii* isolation protocol derived from a method originally developed for other plants (Doyle & Doyle 1987, Kathiresan et al 1993). Modifications were made to minimize polysaccharides co-isolation and to simplify the procedure for processing large number of sample. The protocol optimized for RAPD proved to be inexpensive with relation to the use of Primer, quality of DNA, usage of dNTPs, Taq Polymerase and the reaction volume. Thus the protocol derived for both genomic DNA isolation and RAPD is genus independent efficient, inexpensive, simple, rapid and yields pure DNA amplifiable by PCR as indicated by the results of the RAPD technique. The isolated DNA would be suitable for further downstream application.

Materials and Methods

Plant Material

For isolation of pure genomic DNA, the young and tender leaves of *Plectranthus forskohlii* were collected from Sirupakkam in wild nature condition. After washing the plant tissue with sterile water and subsequently with 70 per cent alcohol, 1g of fresh leaf tissue of species was taken and then it was chopped into fine pieces and subjected to genomic DNA isolation. Simultaneously leaves of the same species were dried in an electric oven at 60^0C for 24 hrs and 1g of dry leaves were processed for extraction of genomic DNA. Genomic DNA was extracted from both fresh and dry leaves by adopting the modified CTAB method outlined by Doyle and Doyle 1987 without using liquid nitrogen. Genomic DNA yield was expressed as µg DNA per mg of leaves tissue.

Testing DNA Isolation methods

Method 1: HipurA Plant Genomic DNA extraction Kit (Himedia, Cat#MB502-PR)

Method 2: Described by Doyle and Doyle 1987. Modified CetylTrimethyl Ammonium Bromide (CTAB) extraction protocol was used. Which was applied in many palnt species. Extraction buffer in this protocol was included: 2% CTAB, 100mM TrisHCl, 2% PVP, 1.4M NaCl, 20mM EDTA (pH: 8.0), 2% B-mercapto ethanol, chloroform : Isoamyl alcohol (24:1), Isopropanol, RNase, 70 % ethanol, TE buffer (10mM TrisHCl, 1mM EDTA pH:8.0), immediately prior to use.

Method 3: Described by Sunil Kumar Sahu et al 2012. Suspension buffer(pH 8.0) in this protocol was included: 50mM EDTA, 120 mMTrisHCl, 1M NaCl, 0.5 M Sucrose, 2% Triton-X 100 and 0.2% b mercapto ethanol (to be freshly added just before use), High salt TE buffer (0.5 M NaCl, 10mM Tris-HCl, 1mM EDTA(pH:8.0).

Suspension buffer solution and TE buffer for modified DNA extraction Sunil Kumar Sahu were prepared according to Table 1.

Modified DNA extraction method of Sunil Kumar Sahu et al 2012 was as follow:

(1) Preheat suspension buffer in water bath at 65^0C. Grind 1g of young leaves to fine powder in ice cold condition in the presence of 100mg PVP (Poly Vinyl Pyrrolidone) by using pre chilled mortar and pestle (-40^0C/-80^0C) (2) Transfer the content to 2 mL micro centrifuge tubes and suspend in two volumes of suspension buffer (3) Invert and mix gently and incubate at 60^0C for 30 min (4) Centrifuge the suspension at 10,000 rpm for 10 min at room temperature. (5) Add 1.5 mL of extraction buffer and incubate at 60^0C for 30 min (6) Centrifuge at 10,000 rpm for 10 min at room temperature (7) Carefully transfer the aqueous phase into a new tube (8) Add double volume of chloroform:Isoamyl alcohol (24:1) and invert gently 10 to 15 times and centrifuge at 10,000 rpm for 10 min (9) Add double volume of chilled isopropanol and keep at -20^0C for one hour to precipitate the DNA (10) Centrifuge at 10,000 rpm for 10 min and discard the supernatant (11) To the pellet, add 70% chilled ethanol and spool out the pellet carefully and centrifuge again at 10,000 rpm for 10 min (12) Discard the supernatant and vacuum dry or air dry the pellet at room temperature (13) Add 100µl of high salt TE buffer (14) Add 3µl RNase and keep at 37^0C for 30 min(14) Add 3 M Sodium acetate (15) Spool out the DNA, wash in 70% ethanol, air or vacuum dry (16) Add 30 to 50 µl (depending upon the pellet) of TE buffer to dissolve the precipitate (17) Store at -20^0C/-40^0C till further use.

Qualitative and Quantitative analysis of Extracted DNA

The yield of DNA per gram of fresh and dry leaf tissue extracted was measured using a Nano photometer (Implen, P360 Version 1.2.0) at 260nm. The purity of DNA was determined by calculating the ratio of absorbance at 260nm to that of 280nm. DNA concentration and purity was also determined by running the samples in 0.8% agarose gel based on the intensities of band when compared with the lambda DNA marker (Used to determine the concentration).

Optimization of Random Amplified Polymorphic DNA (RAPD) Reaction

The PCR amplification reaction was carried out with ten Oligo nucleotide Operon primers from W and A series obtained from GeNei (Bangalore). Each 20 µl reaction volume containing 10mM TrisHCl (pH 8.3), 2.5mM MgCl2, 25mM dNTPs mix, 0.2µM of each primer, 10x Taq buffer, 1U of Taq DNA Polymerase and 50 ng of template DNA. RAPD-PCR was performed in Master cycler nexus (Eppendorf) for 40 cycles consisting of denaturation at 94^0C for 45 sec, annealing at 38^0C for 50 sec, and extension at 72^0C for 60 sec. The final extension was carried out at the same temperature for 10 min and the hold temperature of 4^0C at the end. The PCR amplified products were electrophoresed on 2%

(w/v) agarose gels, in 1x TAE buffer at 65 V for 3 hrs and then stained with ethidium bromide (0.5µg/ml). Gels with amplification fragments were visualized and Photographed under UV gel documentation system (Alpha Innotech). Lambda DNA was used as molecular marker (GeNei, Bangalore) to know the size of the fragments.

Result and Discussion

Choice of the material

Proper choice of the leaf tissue is very important for DNA extraction (Lodhi et al 1994). In this research, leaf tissue collected from wild condition and used for DNA extraction because fresh, young leaf tissue was preferable since it may contain low polyphenolic and terpenoid compounds then older tissue (Rosenthal et al 1979). Generally, mature plant tissue are not preferred for DNA extraction due to the presence of high concentration of polysaccharides, polyphenols and other secondary metabolites (Dabo et al 1993; Zhang et al 2000).

DNA yield

Yield of the DNA extracted by three methods were listed in Table 2. The DNA yield by modified method of Sahu et al were significantly higher than those obtained by HipurA kit method, and standard CTAB method and Sahu et al method. DNA extracted by using the method of Doyle & Doyle had not yield good quantity and quality from both fresh and dry leaves of *P.forskohlii*. Furthermore, these results were in agreement with the findings of Zeigen (1993). In our research, a high yield of DNA was obtained from both fresh and dry leaf tissues of *P.forskohlii* using modified Sahu et al method. Probably because the young *P.forskohlii* leaves contain less secondary metabolites.

The lowest DNA yield was obtained by the method reported by Doyle & Doyle and HipurA kit method. This result accorded with Ostrowka et al 1998, Abu-Romman 2011 and Doosty et al 1994. By using Doyle & Doyle, Ostrowska 1998 yield was 48-67 µg per gram (equal to 4.8-6.7 µg per 100mg) DNA from *Pinus radiate*, Abu-Rromman got the lowest DNA yield and poor quality from Sage (*Salvia officinalis*) and Doosty et al obtained negligible DNA from *Saturejakhuzistanica*.

Purity

The assessment of the purity of nucleic acid sample in often performed by a procedure commonly referred to as the $OD_{260/280}$ ratio. Although this procedure was first described by Warburg & Christian (Warburg et al 1942) as a means to measure protein purity in the presence of nucleic acid contamination, it is most commonly used today to assess purity of nucleic acid sample (Held et al 2006). A pure sample of DNA has the ratio at 1.8 (Chen et al 2010).

The mean $OD_{260/280}$ ratios obtained to DNA extracted by these three methods HipurA kit, CTAB and Sahu et al, were higher than 1.9. In these three methods RNA disposal was not involved, hence there existed some RNA residues, as determined by the electrophoresis on agarose gel (Figure1, there were clear main bands observed). The mean $OD_{260/280}$ ratios of

CTAB method, HipurA kit method were observed in the ratio between 1.4to1.7. It meansthe extracted DNA was relatively free from RNA and Protein contamination. RNAse was used to remove RNA from DNA in all the three procedure.

Integrity

The integrity presence of high molecular genomic DNA was determined by electrophoresis 0.8 % agarose gel. High molecular DNA bands were obtained from all these three methods while showed bands with smear with the bottom of the lane 1, 2, 3 (Figure 1), demonstrating that the DNA were intact but there existed some RNA or Protein residues.

Functionality

There are at least three main contaminants associated with plant DNA,Polyphenolic compounds, polysaccharides and RNA (Jobes et al 1995). Polysaccharides, which are difficult to separate from DNA (Murray et al 1980), interfere with several biological enzymes such as polymerases, ligases and restriction endonucleases (Shioda et al 1987; Richards 1985). More over Lodhi et al 1994 found that when polysaccharides were not removed, the DNA would not amplify in PCR reaction.

The PCR reaction using ten randomized Operon Oligonucleotide RAPD primers (Table 3) was carried out to compared DNA extracted quality (Figure2). The amplification of *P.forskohlii* DNA was observed only on the OPW series primers. There is no amplification for OPU series of Primers. As the observed DNA extracted from the selected method (Sahu et al, had good amplification and also had the good banding pattern (Figure2).

There are two different viewpoints on the effect of RNA residue. Some researchers hold the opinion that contaminants like RNA often inhibit restriction endonucleases digestion and / or PCR amplification (Couch et al 1990; Guillemaut et al 1992; Richards et al 1994). There is also new data indicating that RNA contamination can reduce the effectiveness of many enzymatic process (Storts 1993; Yoon et al 1993; Mejjad et al 1994). Furthermore, the RNA degrades at high temperature in the presence of magnesium ions and the release nucleotide incorporation in the PCR condition.

While other argues that the presence of the RNA in DNA extracted is not major problem as this usually does not interfere with PCR or restriction digestion (Murray et al 1980; Vinod 2004). Because RNA is, by nature, transient and unstable unlike DNA. RNA is ubiquitously degraded with striking efficiency in all cells (Houseley et al 2009). Much of the RNA is cut by ribonucleases or RNAses that are released when the cells are broken open and the rest will not last in an environment outside the cell and will degrade anyways even without RNAse.

Conclusion

In this study, three methods for used for isolating DNA from *P.forskohlii* were compared and analysed from the following perspectives: yield of DNA, the purity of DNA acquired, intactness, and functionality. All the three methods compared in this study turned

out to be suitable to extract DNA from *P.forskohlii*. In summary, the conclusions in this research are as follows:-

1. The yield of DNA from *P.forskohlii* by Sahu et al method are significantly higher than those obtained by the CTAB and HipurA kit method.
2. The extraction method had a significant effect on the DNA yield and $OD_{260/280}$ ratio, both fresh and dry leaf tissue of *P.forskohlii*.
3. After evaluating the yield, purity, integrity, functionality among the three methods, the Sahu et al method was considered an ideal protocol to isolate DNA from *P.forskohlii* by using both fresh and dry leaves.
4. Besides, the quality and quantity of the DNA extracted by this method were high enough to perform hundreds of PCR-based reactions and also to be used in other DNA manipulation techniques such as RFLP, AFLP, ISSR, Restriction digestion, Southern Blot and Cloning.

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Table 1. Different Concentration of Modified method of Sahu et al

S.No	Name of the Solutions	Standard Concentration	Modified Concentration
1	**Suspension Buffer**		
	EDTA	50mM	20mM
	Tris –HCl	120mM	100mM
	NaCl	1M	1.5M
	Sucrose	0.5M	1M
	Triton-X 100	2%	2.5%
	B-mercapto ethanol	0.2%	1.5%
2	**High Salt TE Buffer**		
	Nacl	0.5M	1M
	Tris-HCl	10mM	20mM
	EDTA	1mM	2mM

Table 2. Comparison of Quality and Quantity of genomic DNA isolated from fresh and dry leaves of *P.forskohlii*

S. No	Methods	Fresh leaves		Dry leaves	
		DNA yield (µg/g)	A260/A280	DNA yield (µg/g)	A260/A280
1	Commercial Kit	1.57±0.04	1.45±0.07	1.47±0.26	1.33±0.08
2	Standard CTAB	22.14±1.85	1.72±0.03	17.74±1.94	1.65±0.07
3	Sahu et al Method	**89.13**±2.81	**1.93**±0.07	**64.56**±0.83	**1.85**±0.07

The results are mean of triplicates determination ± standard deviation.**Data are means** ± SD (n=3).

Table 3. Details of Primers using RAPD-PCR analysis on DNA from *P.forskohlii*by using Sahu et al Method.

S.No	Primer	Sequences
1	OPW 06	5’-AGGCCCGATG-3’
2	OPW 07	5’-CTGGACGTCA-3’
3	OPW 08	5’-GACTGCCTCT-3’
4	OPW 09	5’-GTGACCGAGT-3’
5	OPW 10	5’-TCGCATCCCT-3’
6	OPU 16	5’-CTGCGCTGGA-3’
7	OPU 17	5’-ACCTGGGGAG-3’
8	OPU 18	5’-GAGGTCCACA-3’
9	OPU 19	5’-GTCAGTGCGG-3’
10	OPU 20	5’-ACAGCCCCCA-3’

Absorption spectrum of DNA isolated from Dry leaves of *P.forskohlii* by using Sahu et al Method

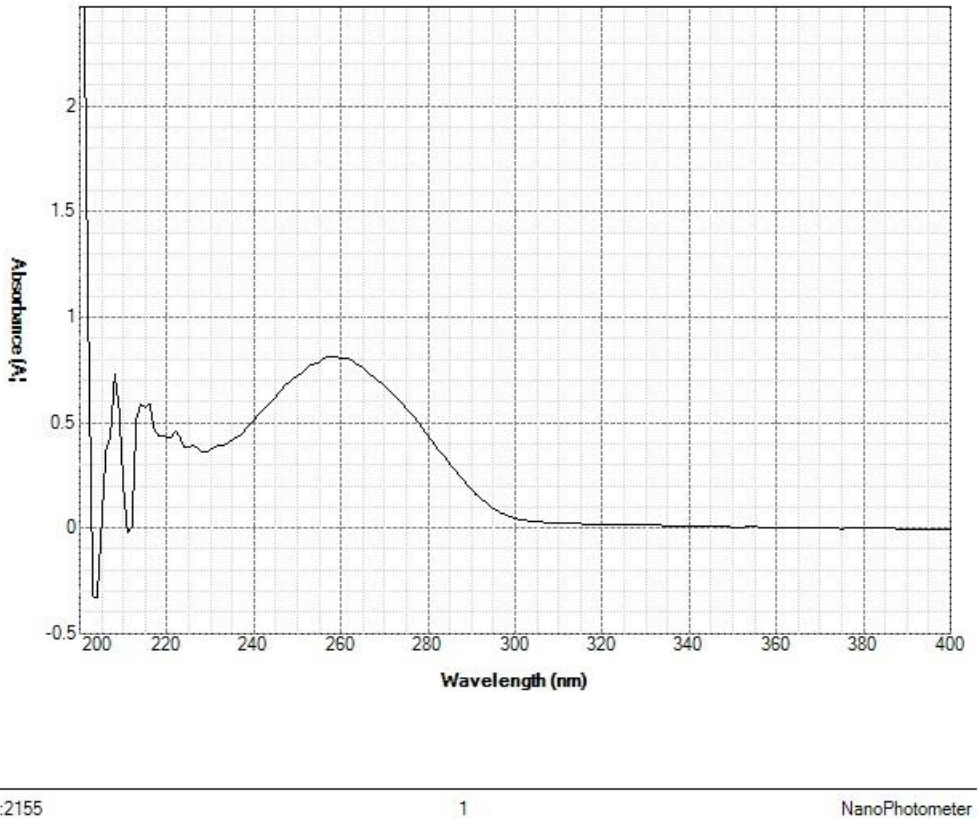
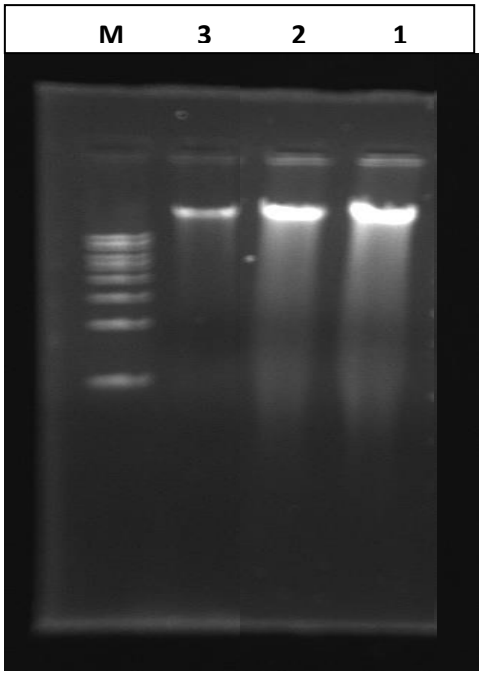


Figure 1. Genomic DNA of *P.forskohlii* resolved in 0.8% of Agarose Gel Electrophoresis



GENOMIC DNA RESOLVED ON 0.8% AGAROSE

M-1Kb DNA Marker

1-HipurA Kit Method

2-Standard CTAB Method

3-Modified Sahu*et al* Method

Figure 2. Polymerase chain reaction using OPW primers on DNA from leaves samples of *P.forskohlii* by using method 3.